

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

HUANG et al.

CASE NO: CL1943 US NA

SERIAL NO: 10/630,248

GROUP ART UNIT: 1762

FILED: JULY 30, 2003

EXAMINER: J. LIN

FOR: MICROPARTICLE-BASED METHODS
AND SYSTEMS AND APPLICATIONS
THEREOF

DECLARATION UNDER 37 C.F.R. § 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In the Non-Final Office Action mailed on July 3, 2006, the Examiner rejected claims 2, 5-17, and 19 under 35 U.S.C. § 103(a) as being obvious over Templeton *et al.*, Langmuir 15:66-76 (1999), in view of Foos *et al.*, Chem. Mater. 14:2401-08 (2002). Specifically, the Examiner asserted that "it would have been obvious to one of ordinary skill in the art at the time of the invention to have used an ethylene glycol oligomer in the preparation of water-soluble gold nanoparticles of Templeton because Foos teaches that an ethylene glycol oligomer can increase the water solubility of a gold nanoparticle."

I, Ming Zheng, declare that water soluble metallic nanoparticles having a mixed monolayer of a capture coating component and a shielding component were reduced to practice in the United States prior to the online publication date of Foos *et al.* (April 19, 2002). Further to this declaration, I attach notebook pages signed by me and witnessed by co-worker Won Chul Suh (Exhibits 1 and 2), with dates redacted, that exemplify the reduced to practice water soluble metallic nanoparticles made through ligand exchange reactions (Exhibit 1) and through direct synthesis reactions (Exhibit 2).

As a person signing below:

Ser. No. 10/630,248
Docket No. CL1943 US NA

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true. I also declare that all statements were made with knowledge that willful false statements, and the like, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and any such willful false statements may jeopardize the validity of either the patent application or any patent issuing thereon.

Respectfully Submitted,

A handwritten signature in black ink, appearing to read "Ming", followed by a long, horizontal, wavy line that extends to the right.

Ming Zheng

12/04/06

Date

TITLE _____ DATE _____

PURPOSE _____

E100993-105

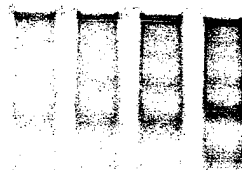
9/6/01 Protein titration experiment with protein staining

Sample 1. 3 μ l An (7/16/01 prep 15:1)2. 3 μ l An + 2 μ l PED 107-6 7/27/01's prep fraction 2, column 2

3. - +4

4. - +6

5. - +8

6. - 110 μ l PET 4/a

6% polyacrylamide gel, 1 hr, 90V

2 well gel

9/11/01 Testing Xueying's ligand exchange samples

Xueying made An-GSH particles last Thursday

Today he did the following test

1. An-GSH + THF

2. An-GSH + EG-SH in H₂O

3. - - - - - in THF

1% TBE, 90V, 40 min

4% TBE, 90V, 40 min

EXPERIMENTER

Ming

DATE

9/11/01

WITNESSED BY

Lian Chao Shi

DATE

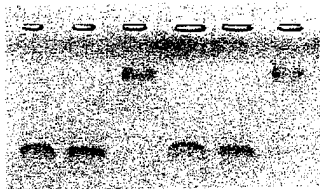
9/11/01

TITLE _____ DATE _____

PURPOSE _____

E 103203- 1

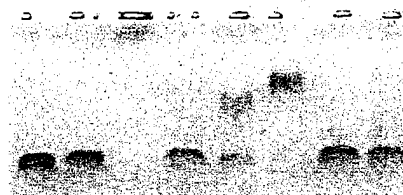
11/15/01



1% TBE, 90 v, 32 min

1. 2 ul of 11/14/01, Au-EG/GSH 1:5
2. 1+ 6 ul PET41a (GST)
3. 1+ 6 ul PED107-6 (GST-ZFP)
4. 2 ul of 11/14/01, Au-EG
5. 1+ 6 ul PET41a (GST)
6. 1+ 6 ul PED107-6 (GST-ZFP)

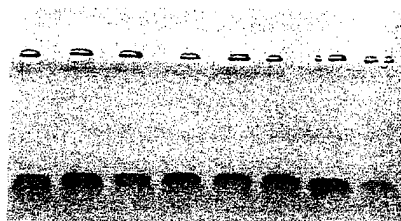
[protein] ~ 1 mg/ml



1% TBE, 90 v, 32 min

1. 1 ul of 9/11/01, Au-EG/GSH 1/6, 40-50% fraction
2. 1+ 6 ul BSA
3. 1+ 6 ul Lysozyme
4. 1+ 6 ul Streptavidin
5. 1+ 6 ul PET41a (GST)
6. 1+ 6 ul PED107-6 (GST-ZFP)
7. 1+ 6 ul PED107-6 (10/22/01's, ~0.2 mg/ml)
8. 1+ 6 ul PED107-6 (10/22/01's, dialyzed, <0.2 mg/ml)

All other proteins ~ 1 mg/ml



1% TBE, 90 v, 32 min

1. 2 ul Au-EG/GSH 5:1
2. 1+ 6 ul PET41a (GST)
3. 2 ul of 11/14/01, Au-EG/GSH 1:1
4. 3+ 6 ul PET41a (GST)
5. 2 ul of 11/14/01, Au-EG/GSH 1:5
6. 5+ 6 ul PET41a (GST)
7. 1 ul of 9/11/01, Au-EG/GSH 1/6
8. 8+ 6 ul PET41a (GST)

11/16/01

PI for a few proteins:

Zif268, $pI=9.9$	10.9 charge at pH 7	90 a.a.
NRE, $pI=9.8$	10.9 charge at pH 7	90 a.a.
PET41a, $pI=6.4$	-4.0 charge at pH 7	285 a.a.

EXPERIMENTER

Ming

DATE 11/16/01

WITNESSED BY

Ken Chelliah

DATE 3/14/02

Ethylene Glycol Monolayer Protected Nanoparticles for Eliminating Nonspecific Binding with Biological Molecules[†]

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Nanoparticles and their hybrid with biological molecules are recognized to have potential applications in electronics, optics, genomics, proteomics, and biomedical and bioanalytical areas.^{1–6} The usefulness of the hybrid materials in many occasions depends on how well one can achieve rational design based on specific binding between inorganic nanomaterials and biological molecules. It is generally recognized that the surfaces of many metallic nanoparticles, such as Au, Ag, Pt, and Cu, are charged,⁷ which cause nonspecific binding with biological molecules via electrostatic interactions. Alkanethiol protected nanoparticles reduce the electrostatic interactions, but induce hydrophobic interactions. In addition, alkanethiol protected nanoparticles are not soluble in water, which makes them incompatible with biomolecules. Nonspecific binding between nanoparticles and biomolecules is a fundamental issue that is not well addressed in the published literature.⁸

In this paper, we demonstrate a new approach to eliminate nonspecific interactions between nanoparticles and proteins by synthesizing ethylene glycol protected gold nanoparticles. Ethylene glycol type molecules are water soluble and neutral in charge and have been demonstrated to resist protein binding on flat gold surfaces.⁹ These early successes prompted us to use ethylene glycol type molecules for nanoparticles. However, the synthesis of an ethylene glycol monolayer on a flat gold surface is very different from that on nanoparticles. Two examples have been demonstrated for preparing poly(ethylene glycol) (PEG) protected gold nanoparticles.¹⁰ It is well understood that poly(ethylene glycol) chains form random coils on a nanoparticle surface. When a ligand is introduced onto the surface of the nanoparticle, the ligand can be easily shielded by the polymer coils, preventing programmable assembling with biological molecules. The ideal surface structure should be a densely packed ethylene glycol monolayer with a well-defined length that is inert to biological binding. In the meantime, a functional ligand can be introduced so that it is accessible to targets, such as protein, cell, etc., for specific binding. To achieve this goal, a short chain with a well-defined length is preferred to long polymer chains for coating nanoparticles.

Very recently, Foos et al. reported the synthesis of short ethylene oxide chain protected gold nanoparticles by the ligand exchange reaction.¹¹ The procedure involved the synthesis of hexanethiol (C6) protected gold nanoparticles followed by two steps of replacing C6 with ethylene glycol thiol molecules. There was also a report on the direct synthesis of tetra(ethylene glycol) protected gold nanoparticles in the aqueous phase with a very low molar ratio of reducing agent (NaBH₄) and tetrachloroauric acid, resulting in partial reduction.¹² With an increasing amount of NaBH₄, the reaction increased aggregation. The yield is very low. Here, we report a direct synthesis of ethylene glycol (HS-(CH₂CH₂O)_n-CH₃, *n* = 2, 3, and 4) protected gold nanoparticles in a mixed solvent of methanol and water by utilizing the NaBH₄ reduction

method, with much improved yield.¹³ The key for this synthesis is the control of water content in the reaction mixture. It was found that water content from 9% to 18% (v/v) is optimized for the formation of stable and water-soluble nanoparticles.

Di-, tri-, and tetraethylene glycol thiol molecules (HS-(CH₂-CH₂O)_n-CH₃, *n* = 2, 3, and 4) were synthesized and purified in our lab.¹⁴ Synthesis of di-, tri-, and tetra(ethylene glycol) monolayer protected gold nanoparticles (Au-S-EG₂, Au-S-EG₃, and Au-S-EG₄) was achieved by controlling the addition of water into the reaction mixture.¹⁴ When the water content was either higher than 18% or lower than 9%, the reaction mixtures began to form agglomerates. It was also noticed that the reaction mixture became aggregated if the reagents contained a very small amount of impurity, such as HS-(CH₂CH₂O)_n-OH (*n* = 2, 3, or 4). The mechanism for the critical water concentration was not investigated. When diluted, these directly synthesized ethylene glycol protected gold nanoparticles became red/purple and clear. The directly synthesized ethylene glycol gold nanoparticles were found to be very stable in aqueous solution and could withstand high salt concentration (1 M NaCl). After 9 months of storage in aqueous solution, no agglomeration was seen. Lyophilized particles could be readily redissolved in water to form a clear red/purple solution. In addition, Au-S-EG_n (*n* = 2, 3, or 4) was found to be very soluble in most organic solvents, such as acetone, methanol, DMSO, chloroform, DMF, and THF. In contrast, our experimental results showed that the indirectly synthesized Au-S-EG_n (*n* = 2, 3, or 4) nanoparticles¹³ by the replacement reaction with tiopronin protected gold nanoparticle (Au-Tp) could not be redissolved after centrifuging purification and drying, and the aggregation was also seen after storage in aqueous solution for several weeks.

The NMR spectra of both the free EG₄-SH molecule and the Au-S-EG₄ nanoparticle are attached in the Supporting Information. The disappearance of the -SH proton signal (t, 1.50–1.56 ppm) in the Au-S-EG₄ nanoparticle clearly supports that the gold nanoparticle is bonded with the EG₄-S- monolayer. The chemical shifts and the integrals of the rest protons confirm that the bonded ethylene glycol molecules have the same structure as the free ones. The average size of the nanoparticles is about 3 nm, as confirmed by the TEM measurement.¹⁴

Protein binding to Au nanoparticles can be conveniently monitored by gel electrophoresis, because the protein-nanoparticle complex is expected to migrate differently than the free Au particles. Protein binding reactions were done by mixing 10 μL of ~0.1 mM Au particles with 1 μL of 10 mg/mL protein in sodium phosphate buffer (50 mM, pH 7.3). After incubation at room temperature for 10 min, the entire reaction mixture was loaded on a 1% agarose gel.¹⁴ Gel electrophoresis results are shown in Figure 1. When Au-Tp particles were mixed with lysozyme and BSA, different degrees of nonspecific binding were observed, as indicated by the band-shifts. When the same experiment was done with Au-S-EG₄ particles, no change in the particle mobility was observed. These

[†] Publication No. 8392.

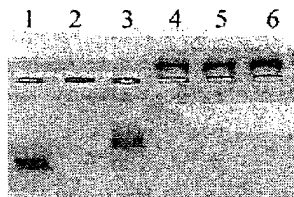


Figure 1. Gel image of protein binding assay. Eleven microliters of protein/particle binding reaction mixture was loaded onto a 1% agarose gel, and electrophoresis was run for 20 min at 90 V constant voltage. Lane 1: 10 μ L of Au-Tp at ~ 100 μ M. Lanes 2 and 3 are the same amount of Au particles mixed with 1 μ L of lysozyme and BSA, respectively. Protein concentrations are all 10 mg/mL. Lanes 4–6 are similar to lanes 1–3, except that Au-EG₄ particles were used.

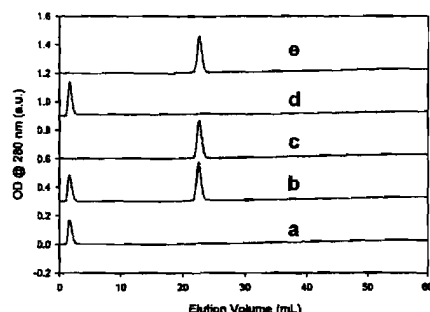


Figure 2. Strong cation exchange chromatography for Au-Tp and Au-EG₄ binding with lysozyme. (a) Au-EG₄; (b) Au-EG₄ + lysozyme; (c) lysozyme; (d) Au-Tp; and (e) Au-Tp + lysozyme.

results clearly showed that the Au-S-EG₄ does not bind to either the very basic protein lysozyme or the very acidic protein BSA.

The most dramatic contrast was provided by the binding between Au particles and lysozyme. Addition of lysozyme into the Au-Tp particle solution causes an immediate color change from pinkish red to blue, indicating that Au particles form aggregates, presumably because the positively charged lysozyme (pI = 11) molecules cross-link negatively charged Au-Tp particles ($-\text{COOH}$). Centrifugation of the Au-Tp/lysozyme reaction mixture resulted in a clear/colorless supernatant, indicating that no soluble Au-Tp particles remained in the supernatant. The cation exchange column chromatography confirmed that only lysozyme proteins were present in the supernatant, as shown in Figure 2. However, when lysozyme was mixed with Au-S-EG₄ solutions, no color change happened in the solution or the supernatant after centrifugation of the Au-EG₄/lysozyme mixture. The reaction mixture was eluted from the cation exchange column as two separate species corresponding to lysozyme and Au-S-EG₄ particles. This result indicates that there is no binding reaction occurring between Au-S-EG₄ and the lysozyme protein. Also tested were cytochrome C, ribonuclease A, and myoglobin. In all cases, no binding was observed between Au-S-EG₄ and the tested proteins, but a varying degree of binding between Au-Tp and those proteins was seen. The Au-EG₄ nanoparticle was also run through an anion exchange column. The Au-S-EG₄ nanoparticles were eluted at the void volume from both cation and anion exchange columns, indicating that the surface of Au-S-EG₄ particles is charge neutral and does not bind to either

column. Consistent with this, we also found that Au-S-EG₄ particles do not bind to either total cellular RNA or chromosomal DNA, both of which are highly negatively charged.¹⁴

In conclusion, we discovered that with the water content optimized in the range of 9–18% in the reaction mixture, di-, tri-, and tetra(ethylene glycol) protected gold nanoparticles Au-S-EG_n ($n = 2, 3$, and 4) could be directly synthesized. These gold nanoparticles that are bonded with a uniform monolayer with defined length varying from 0.8 to 1.6 nm (from molecular modeling) have great stability in aqueous solutions with a high concentration of electrolyte and organic solutions. Using ion-exchange chromatography and gel electrophoresis, we first demonstrated that these Au-S-EG_n ($n = 2, 3$, or 4) nanoparticles with neutral and hydrophilic surfaces have complete resistance to protein nonspecific interactions. These types of nanoparticles provide a fundamental starting material for designing hybrid materials composed of metallic nanoparticles and biomolecules. One of the advantages for the direct synthesis method is that it allows the synthesis of a mixed monolayer of ethylene glycol and a functional ligand to eliminate nonspecific interactions and provide specific interactions in the same time.¹⁵ From the aspect of industrial applications, these biologically inert gold nanoparticles will have commercial utilities in electronic and biomedical applications. Some of these works are underway.

Supporting Information Available: Synthesis of EG_n-SH ($n = 2, 3$, or 4), synthesis of the Au-EG₄ nanoparticle, synthesis of Au-S-EG_n ($n = 2, 3$, or 4) through replacement with the tiopronin protected Au nanoparticle, gel electrophoresis experiment, ion exchange chromatography, ¹H NMR spectra of free EG_n-SH and the Au-S-EG₄ nanoparticle, TEM image of Au-EG₄, and gel electrophoresis assay of Au-EG₄ binding with DNA and RNA (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

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- (14) See Supporting Information.
- (15) Manuscript in preparation.

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